EXHIBIT

D

Gene Therapy (1996) 3, 065–067

Gene targeting of CFTR DNA in CF epithelial cells

K Kunzelmann[1,2], J-Y Legendre[3,4], DL Knoell[3,5], LC Escobar[1], Z Xu[1] and DC Gruenert[1,6]

[1]Cardiovascular Research Institute, Gene Therapy Core Center, and Cystic Fibrosis Research Center, [3]School of Pharmacy and [6]Departments of Laboratory Medicine and Stomatology, University of California, San Francisco, California 94143, USA

A goal of cystic fibrosis (CF) gene therapy is correction of the mutant CF transmembrane conductance regulator (CFTR) gene with wild-type (wt) DNA sequences to restore normal CFTR protein and function. Experiments with wtCFTR cDNA expression vectors have shown that the Cl ion transport phenotype associated with CF can be corrected to resemble that in normal cells. An alternative to cDNA-based gene therapy strategies is one that corrects endogenous mutant sequences by targeted replacement with the wt homologue. To test whether such a strategy was feasible, a small fragment homologous replacement (SFHR) strategy was used to replace specific genomic sequences in human epithelial cells. Small fragments of genomic wtCFTR DNA were transfected into transformed CF epithelial cells. Replacement by exogenous CFTR DNA at the appropriate genomic locus and its expression as mRNA was indicated by: (1) allele-specific polymerase chain reaction (PCR) amplification of genomic DNA and mRNA-derived cDNA; and (2) hybridization of PCR products with allele-specific probes. In addition, the functional activity of CFTR protein was determined by whole cell patch clamp. Southern hybridization and patch clamp analyses suggested that approximately 1 in 100 CF cells underwent a homologous replacement event that resulted in intact Cl transport.

Keywords: *gene therapy; homologous replacement; small DNA fragments*

Introduction

Isolation and characterization of the *CFTR* gene[1,2] has been crucial for understanding the biochemical mechanism(s) underlying CF pathology. A 3-bp, in-frame deletion eliminating a phenylalanine at codon 508 (ΔF508) of CFTR protein is the most common CF mutation and has been found in about 70% of all North American CF chromosomes.[3,4] This and other *CFTR* gene mutations result in the cAMP-dependent Cl ion transport defect which gives rise to the CF phenotype.

Correction of defective Cl ion transport is the basis for all CF gene therapy strategies. Initial *in vitro* studies showed that introduction of recombinant viral vectors carrying *wtCFTR* cDNA into CF epithelial cells corrects this Cl ion transport defect.[5–8] Gene therapy strategies have used adenovirus vectors for transfer of *wtCFTR* cDNA into the human airway epithelium[9–12] and liposome-mediated gene transfer to human nasal mucosa.[13] Thus far, the adeno-associated virus (AAV) system has been shown to be effective for transfer into cultured CF airway epithelial cells[7] and rabbit airways[14] and is now entering into a clinical trial.

While these cDNA-based approaches are an important first step toward development of CF gene therapy protocols, there are numerous drawbacks. The first is a significant immune response following exposure to adenoviral vectors which leads to inflammation in the infected area.[10,12,15,16] The second is that under certain circumstances, overexpression of *wtCFTR* cDNA can be toxic *in vitro* and *in vivo*.[17,18] Eukaryotic expression vectors carrying the *CFTR* cDNA are independent of the regulatory systems that control cellular levels of CFTR. As a result, transcription of *CFTR* cDNA may activate metabolic and physiologic feedback mechanisms that are not compatible with normal function and/or survival. Finally correction appears to be transient[10–13,15,16] and may not completely resolve the Cl^- and Na^+ transport abnormalities also associated with CF.[19]

Since solutions to these problems remain elusive, it is unclear whether the cDNA-based gene therapy strategies will result in the normal phenotype necessary to treat CF individuals. It may therefore be necessary to develop an alternative gene therapy approach. One possibility would be direct correction of the genomic lesion by gene targeting. Recent studies exploring gene targeting have shown correction of episomal DNA either with oligonucleotides[20–22] or by using replication-defective adenovirus vectors.[23]

We tried SFHR as a targeted gene replacement strategy to correct the *ΔF508* mutation. Small (491 bp) fragments of genomic *CFTR* DNA were introduced into CF cells. To initiate SFHR, cells were exposed to approximately 10^9 DNA fragments per cell using liposome or polyamidoamine vehicles to transfer the DNA. Some studies were carried out with rec A coated fragments as there is evidence that homologous pairing in human DNA can be enhanced by the coating of single strand DNA (ssDNA)

Correspondence: DC Gruenert, Gene Therapy Core Center, Cardiovascular Research Institute, Cystic Fibrosis Research Center, University of California, San Francisco, CA 94143–0911, USA

Present addresses: [2]Institute of Physiology, Albert-Ludwigs University, Hermann Herder Strasse 7, D-79106 Freiburg, Germany; [4]Pharmacy Research and Development, Hoffmann-LaRoche Ltd, Grenzacher Strasse 124, CH-4002 Basel, Switzerland; [5]Department of Pharmacy, Ohio State University, Columbus, Ohio 43210, USA

Received 6 June 1996; revised 19 July 1996; accepted 1 August 1996

fragments with the bacterial recombinase, rec A.[23] Analysis of genomic DNA, cytoplasmic RNA, and Cl ion transport indicated homologous replacement and correction of the CF Cl transport defect.

Results

PCR analysis of the genomic cell-derived DNA and cytoplasmic RNA was carried out with both allele-specific primers and primers outside the region of homology (Figure 1). To ensure that the PCR analysis of the DNA was only from the genomic locus comprising the region of homology, in either case at least one primer was outside the 491-bp region of homology defined by primers CF1 and CF5 (Figure 1a) (Tables 1 and 2). Reverse transcription PCR (RT-PCR) of the cytoplasmic RNA was carried out where amplification always crossed intron/exon boundaries (Figure 1b). Electrophysiological analysis was carried out specifically to measure Cl currents and membrane voltage was monitored continuously. The whole cell Cl current was measure in intervals by stepwise clamping of membrane potential to ±30 mV in increments of 10 mV.

DNA analysis

PCR analysis of cellular DNA indicated that SFHR had occurred in CF cells transfected with *wtCFTR* fragments. ΣCFNPE14o– DNA was analyzed 4 days after transfection with the dendrimer–DNA complex by allele-specific Southern hybridization of PCR products derived from the genomic exon 10 locus with primers (CF1B/CF6) (Figure 2). These primers are outside the homologous region defined by the transfection fragment. The hybridization analysis shows genomic *wtCFTR* sequences were present in cells transfected either by gramicidin S-DNA–lipid (GS) or with the Starburst dendrimer–DNA (SD) complexes (Polysciences, Warrington, PA, USA) (Figure 2). Replacement frequency values were calculated after densitometric readings for *ΔF508* signals (bottom panel) in all lanes. By comparing hybridization of N and

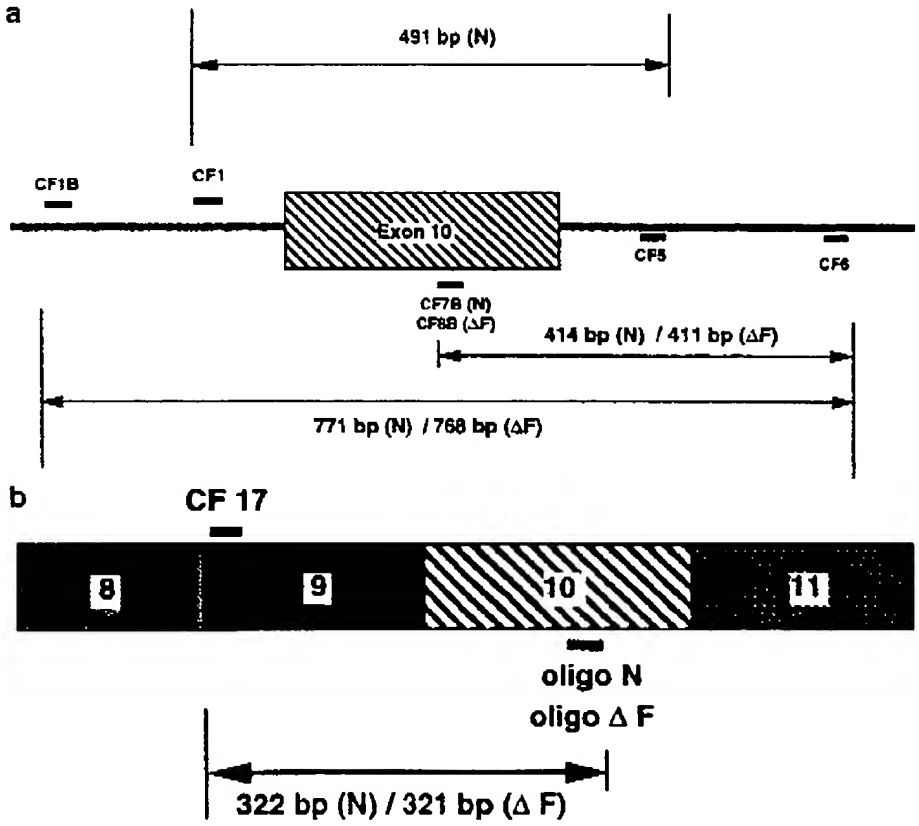

Figure 1 (a) The region of homology defined for the exogenous fragment is a 491-bp DNA stretch of DNA defined by PCR primers CF1,CF5. Cellular DNA and RNA from transfected cells were isolated and analyzed for evidence of homologous replacement. Several different approaches were taken for the analysis of DNA. Both allele-specific Southern blot hybridization of PCR products and allele-specific PCR amplification of genomic DNA were used to assess homologous replacement. To ensure that the genomic locus containing the homologous region was specifically amplified, at least one PCR primer was outside the region of homology. In a successful transfection, amplification with primers CF1B,CF6 (both are outside the homologous region) results in 771-bp and 768-bp fragments (3 bp are deleted for ΔF508 CFTR DNA). Detection of wtCFTR within this population of PCR products was accomplished by Southern hybridization with DNA probes specific for wt sequences. Allele-specific amplification of genomic DNA was carried out with primers CF7B,CF6 (414-bp wtCFTR fragment) or CF8B CF6 (411-bp ΔF508 fragment). (b) Analysis of RNA for wtCFTR exon 10 sequences was carried out with primers that span intron/exon boundaries and assure that only CFTR mRNA-derived cDNA is amplified. Primers in exon 9 (CF17) and allele-specific (wt, oligo N or ΔF508, oligo ΔF) primers in exon 10 were used. Amplification with primers CF17 N, yields a 322-bp normal DNA fragment only if transcription of homologously recombined DNA has occurred. A 321-bp fragment would be generated for the ΔF508 mutation present in the parental cell line (see Table 2 for PCRC conditions).

Table 1 PCR primers and probes for SFHR analysis

Primer	Sequence	Location
CF1 (S)	5'-GCAGAGTACCTGAAACAGGA-3'	Intron 9
CF1 B (S)	5'-CCTTCTCTGTGAACCTCTATCA-3'	Intron 9
CF5 (A)	5'-CATTCACAGTAGCTTACCCA-3'	Intron 10
CF6 (A)	5'-CCACATATCACTATATGCATGC-3'	Intron 10
CF7B (A)	5'-CCATTAAAGAAAATATCATCTTTGG-3'	Exon 10
CF8B (A)	5'-CCATTAAAGAAAATATCATTGG-3'	Exon 10
CF17 (S)	5'-GAGGGATTTGGGGAATTATTTG-3'	Exon 9
oligo N (A)	5'-CACCAAAGATGATATTTTC-3'	Exon 10
oligo ΔF (A)	5'-AACACCAATGATATTTTCTT-3'	Exon 10

The nucleotide sequence of *CFTR* DNA primers are as described in the text and were derived from published data.[34,35] Sense (S) and antisense (A) primers are as indicated.

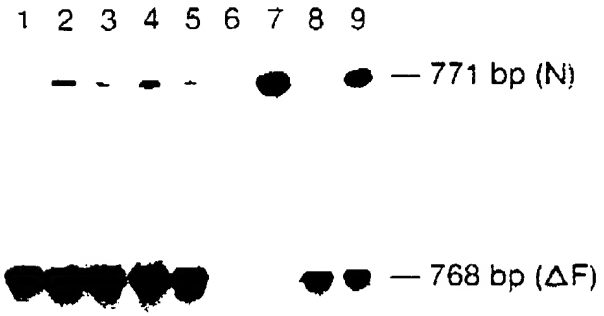

Figure 2 Autoradiographic analysis of CF1B/CF6 PCR products (771 768 bp) of DNA from ΣCFNPE14o– cells transfected with 491-nt fragments as a dendrimer–DNA complex. The PCR product in each lane is from the following: lane 1, nontransfected control cells; lanes 2 and 4, cells transfected with uncoated DNA fragments; lanes 3 and 5, cells transfected with rec A coated DNA; lane 6, H_2O; lane 7, from non-CF (N/N) lymphocytes; lane 8, ΔF508 homozygote (ΔF/ΔF) lymphocytes; lane 9, ΔF508 heterozygote (ΔF/N) lymphocytes. The upper and lower panels represent hybridization to two separate blots of gels run in parallel. Equivalent volumes of sample were aliquoted from the same PCR reaction for the same lane of each hybridization.

Table 2 PCR fragments

Primers	Fragment size
CF1/CF5[a]	491-bp(N)/488-bp(ΔF)
CF1B/CF6[b]	771-bp(N)/768-bp(ΔF)
CF7B/CF6[c]	414-bp (N)
CF8B/CF6[b]	411-bp(ΔF)
CF17/N[c]	322-bp(N)
CF17/ΔF[c]	321-bp(ΔF)

Different primer pairs and the resulting fragments following amplification.
[a]Primers, 0.4 μM; DNA 50–100 ng; 94°C/60 s, denaturation; 55°C/30 s, annealing; 72°C/30 s with 4 s per cycle increase, extension; Mg^{+2} 1.5 mM; 30 cycles.
[b]Primers, 0.5 μM; DNA 50–100 ng; 95°C/60 s, denaturation; 56°C/60 s, annealing; 72°C/120 s, extension; Mg^{+2} 2.0 mM; 30 cycles with 8 min extension on the last cycle.
[c]Primers, 0.5 μM; 95°C/95 min, denaturation; 51°C/30 s, annealing; 72°C/20 s, extension; Mg^{+2} 0.8 mM; 40 cycles with a 4 s per cycle increase in extension time.

ΔF probes to PCR products (lane 9) derived from heterozygote (N/ΔF) DNA, it was determined that the N probe signal was 1.1 greater than that of the ΔF508. To correct this difference in hybridization efficiency between the two probes, the values for ΔF signals were normalized by multiplying by 1.1. Replacement frequency was determined as described in the Materials and methods. Densitometric analysis indicated a replacement frequency of approximately 3–10%.

ΣCFTE29o– cells (Figure 3a) and CFPAC-1 cells (Figure 3b) also showed site-specific replacement of the *ΔF508* following direct allele-specific PCR amplification of genomic DNA. Cells transfected with the gramicidin S-DNA–liposome complex or the dendrimer–DNA complex were harvested 3 and 10 days after transfection showed the presence of *wtCFTR*.

RNA analysis

Corrected DNA must also be expressed as mRNA to restore CFTR function. Allele-specific RT-PCR analysis of ΣCFTE29o– cells transfected either by electroporation or in a gramicidin S-DNA–lipid complex with the *wtCFTR* fragment confirmed that the cells expressed *wtCFTR* mRNA following transfection (Figure 4). Amplification of genomic DNA is circumvented by using primers that amplify across intron/exon boundaries. Amplified mRNA-derived cDNA from normal, 16HBE14o–, cells (lane 2) and transfected ΣCFTE29o– cells (lanes 6–9) yielded DNA fragments (322 bp) using allele-specific primers with the CF17 oligo N. Amplification of mRNA-derived cDNA from ΣCFTE/con cells only showed a PCR product (321 bp) after amplification with the allele-specific primers, CF17 oligo ΔF primers, but not with the CF17/oligo N primers (lane 3). Two separate electroporation experiments with the 491 nucleotide *CFTR* fragments (lanes 6 and 7) indicated the presence of *wtCFTR* mRNA in the ΣCFTE29o– cells. In addition, RNA from gramicidin S-DNA–lipid-transfected ΣCFTE29o– cells also contained *wtCFTR* mRNA whether or not the 491-nt fragments were coated with rec A (lanes 8 and 9). A zero time control isolation of RNA after transfection with fragment and subsequent amplification did not yield any PCR product (data not shown). These analyses strongly

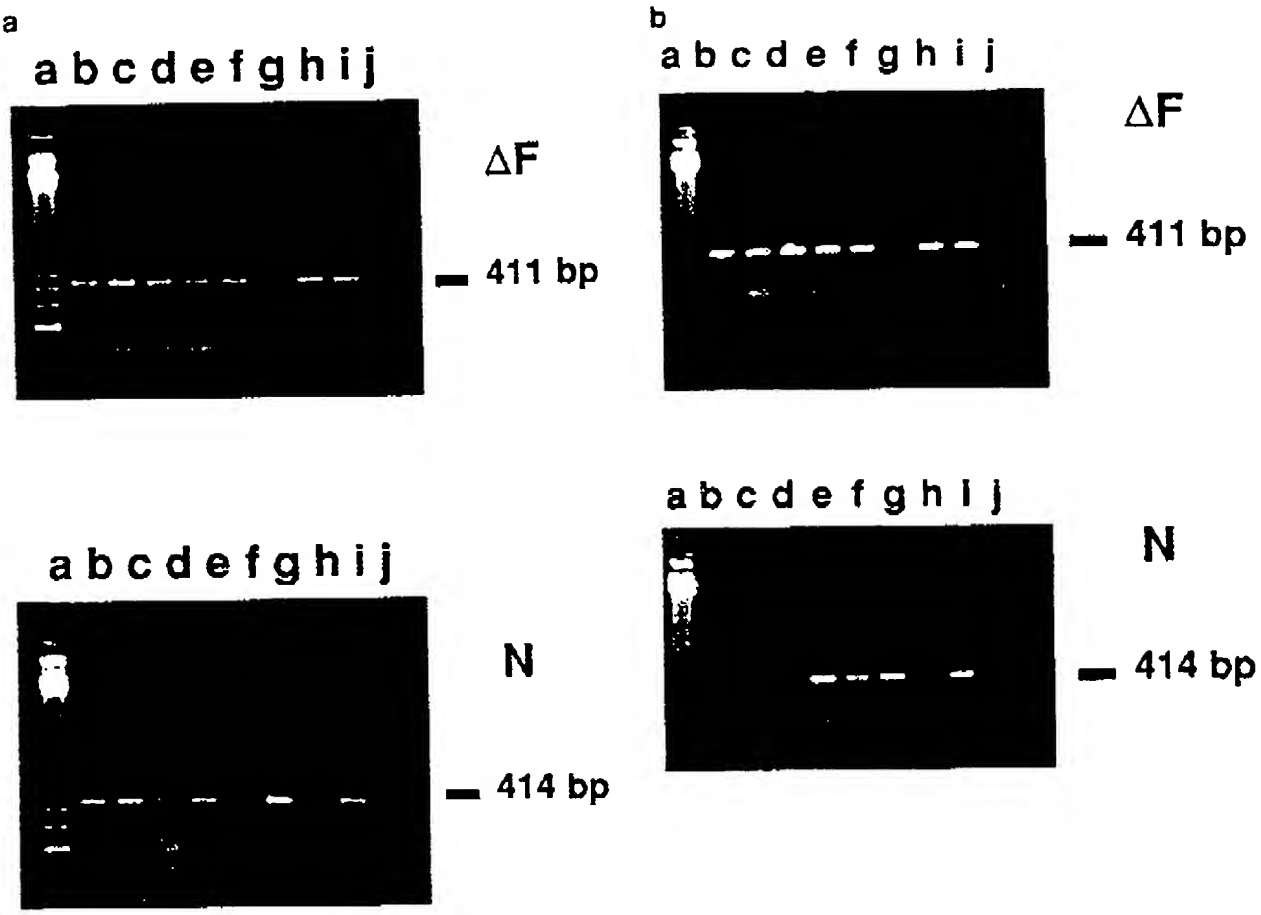

Figure 3 Allele-specific PCR analysis (using primers CFTR/CF6 (N) or CFSB/CF6 (ΔF)). (a) Analysis of ΣCFTE29o− cells transfected with DNA complexed to Starburst dendrimer (lanes b and c) or gramicidin S–lipid (lanes d and e). DNA fragments were uncoated (lanes b and d) or rec A-coated (lanes c and e). Isolated DNA was analyzed for ΔF508 (ΔF, top) and wtCFTR (N, bottom). Controls were of DNA from: nontransfected, lane f cells; non-CF (N/N) lymphocytes, lane g; ΔF508 homozygote (ΔF/ΔF) lymphocytes, lane h; ΔF508 heterozygote (ΔF/N) lymphocytes, lane i; and H_2O, lane j. (b) Allele-specific PCR analysis of CFPAC-1 cells transfected with 491-nt fragments using the Starburst dendrimer–DNA complex. PCR analysis of CFPAC-1 DNA from nontransfected (lane b) and mock-transfected (without DNA fragment) control cells (lanes c and d). Cells transfected with fragment (lanes e and f) and control DNA from the N/N and N/ΔF lymphocytes (lanes g and i) indicated wtCFTR sequences. The ΔF508 homozygote (ΔF/ΔF) lymphocyte control is in lane h. A 123-bp marker was used (lane a) and an H_2O control was in lane j.

suggest expression of genomic DNA sequences replaced at the *CFTR* exon 10 locus with incoming homologous DNA fragments.

Electrophysiological analysis

Patch clamp analysis of ΣCFTE29o− cells transfected with the gramicidin S-DNA–lipid (GS) or the dendrimer–DNA (SD) complex was performed to detect cAMP-dependent Cl transport. The homologous DNA fragment was either naked (GS− or SD−) or rec A-coated (GS+ or SD+). When the results are compared to those from non-CF, 16HBE14o− cells (Figure 5, panel d), the level of whole cell Cl conductance (G_{Cl}) was similar for the responding transfected cells (Figure 5, panel f), while the control nontransfected CF cells failed to show any increase in cAMP-dependent G_{Cl} (Figure 5, panel b). Analysis of 48 ΣCFTE/con cells and 71 of 78 transfected ΣCFTE29o− cells (ΣCFTE/T−) showed no increase in G_{Cl} following exposure to forskolin. However, in seven of 78 transfected cells (9%), forskolin increased Cl conductance (ΣCFTE/T−) (Table 3). In these seven positive cells, whole cell Cl conductance was reversibly increased by forskolin (Table 4) (Figure 5, panel f). Membrane depolarization (ΔPD) was also reversibly increased after reducing the bath Cl concentration to 30 mM (Table 4) (Figure 5, panel e). There was no indication of a cAMP-dependent Cl current immediately after transfection with fragment. The Cl conductance of the unstimulated cells (G_{Con}) at time zero after transfection was 4.3 ± 0.9 nS. The forskolin induced Cl conductance (G_{For}) was unchanged at 4.3 ± 0.8 nS ($n = 5$). Comparable increases in the whole cell Cl conductance were seen in ΣCFTE/con, ΣCFTE/T−, and ΣCFTE/+ cells after exposure to ionomycin (G_{Ion}; 5×10^{-7} M) and after hypotonically induced cell swelling (G_{Hypo}) (Table 4).

Discussion

This study demonstrates homologous replacement of endogenous *ΔF508* CFTR sequences by exogenous

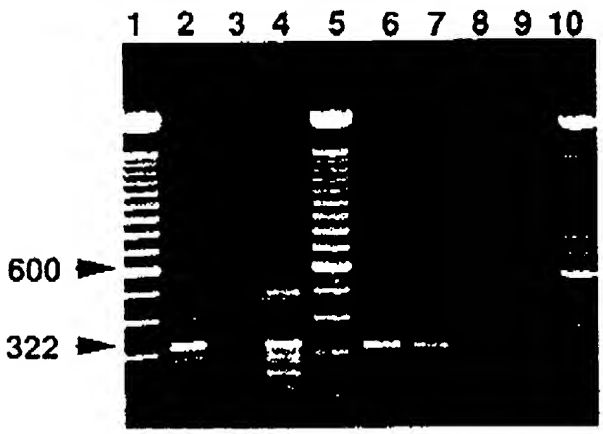

Figure 4 RT-PCR analysis of CFTR mRNA from ΣCFTE29o− cells electroporated with rec A-coated 491-nt fragments (lanes 6 and 7) or with uncoated or rec A-coated DNA in the GS complex (lanes 8 and 9, respectively). Cytoplasmic RNA was isolated 7 days after transfection. A 321-bp PCR product was produced from transfected ΣCFTE29o− cells when the cDNA was directly amplified with primers CF17 ΔF in control cells (lane 4). However, only normal control 16HBE14o− cells (lane 2) and the transfected cells (lanes 6–9) gave a product when amplified with primers CF17 N. No product was observed when cDNA from ΣCFTE/con cells was amplified for wtCFTR sequences (lane 3). Marker DNA (100-bp) is in lanes 1, 5 and 10.

Table 3 Breakdown of ΣCFTE29o− patch clamp analysis

ΣCFTE29o−	Cont	GS/−	GS/+	SD/−	SD/+
Cells (*n*)	48	22	11	15	30
Responses (*n*)	0	3	0	1	3

ΣCFTE29o− cells transfected with either a gramicidin S-DNA–lipid (GS) or the Starburst dendrimer–DNA (SD) complex. The DNA fragment was rec A coated (GS+, SD+) or uncoated (GS−, SD−). Cells were individually analyzed by patch clamp. Responding cells showed a Cl conductance and a change in membrane potential after forskolin stimulation and a reduction in bath NaCl concentration to 30 mM.

wtCFTR DNA in transformed CF epithelial cells. Analysis of *CFTR* mRNA in the transfected cells indicated that the transfected exon sequence is also expressed. In addition, CF cells that have been corrected by homologous replacement display intact cAMP-dependent Cl transport, thereby indicating expression of functional CFTR protein.

Direct administration of small ssDNA fragments, as reported in this study, advances previous homologous recombination studies[5–22,24] and shows that homologous replacement with small genomic DNA fragments can be used to correct naturally occurring *CFTR* mutations in CF epithelial cells. SFHR has an advantage over vector-based homologous recombination strategies,[25,26] because intron sequences are not disrupted by selectable marker gene sequences. This eliminates possible interference of marker gene transcription with that of the targeted gene and predisposes the homologous pairing to be energetically more stable. While the number of cells corrected under these conditions is in the range of 10^{-2}, it should

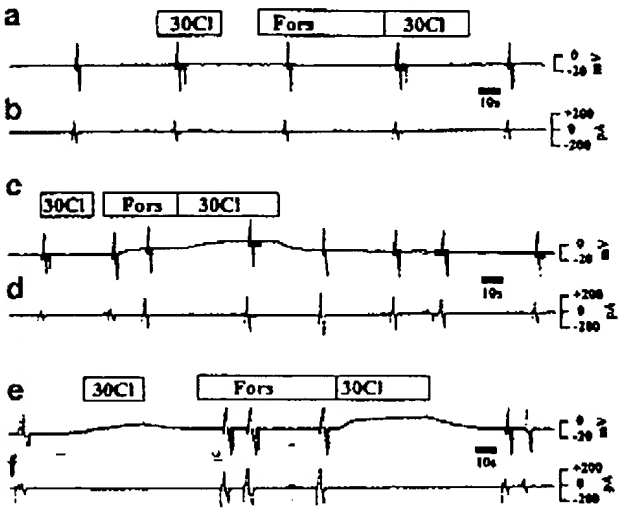

Figure 5 Continuous recordings of three different whole cell patch clamp experiments with ΣCFTE/con (control) (a, b), 16HBE14o− (c, d), ΣCFTE29o−/SD− (e, f) cells. Cell membrane potential was measured continuously (a, c, e: current clamp) and the whole cell current was measured in intervals by voltage clamping to ±30 mV (b, d, f). Forskolin (Fors; 10 μM) had no effect on whole cell current of ΣCFTE/con cells (a) and Cl conductance could not be detected by reduction of the bath Cl concentration to 30 mM (30 Cl; b). Whole cell currents were increased by Fors in 16HBE14o− (d) and in transfected (ΣCFTE29o−/SD−) (f) cells. Moreover, after stimulation a significant increase in the membrane depolarization was observed following reduction of the bath Cl (30 Cl; c, e) and indicates activation of Cl conductance. All effects were reversible.

be noted that the cells are exposed to approximately 10^6 copies of fragment per cell. Thus on a per cell basis, assuming a transfection efficiency of 10^{-1}–10^{-2},[27,28] the frequency of homologous replacement is approximately 10^{-5}–10^{-6}.

The wc patch clamp analysis is further confirmation of SFHR. Within a population of transfected CF cells, a significant subpopulation of cells have had their cAMP-dependent Cl transport defect corrected. The increase in whole cell current in seven of 78 forskolin-treated cells and the depolarization due to the reduction of the bath Cl in each of the seven responding cells, clearly indicates that the whole cell increase was due to activation of a Cl conductance. Precautions were taken to reduce the contribution of K^+ currents to the whole cell current by having CsCl in the pipette filling solution. The continuous recording of cell membrane potential kept the cells at their own membrane potential and reduced artifacts due to cell swelling or voltage clamping. The apparent homologous replacement in 9% of the cells is in reality a maximum frequency in that it assumes that each responding cell represents a single homologous replacement event. However, we can not rule out the possibility that in cases where multiple responding cells were detected (GS− and SD+, Table 3), each responding cell may have been derived from a single cell in which homologous replacement occurred. In this case, the responding cells would, in effect, reflect a minimum of three separate homologous replacement events indicating a frequency of approximately 4% (three in 74 cells). These values show close agreement with a frequency from the densitometric analysis (3–7%). In any event, the degree of homologous

Table 4 Whole cell Cl transport responses

cAMP-dep Cl transport	ΣCFTE/con ($n = 48$)	ΣCFTE/T− ($n = 71$)	ΣCFTE/T+ ($n = 7$)
G_{con} (nS)	3.9 ± 0.8	4.4 ± 0.4	4.2 ± 0.9
G_{For} (nS)	3.9 ± 0.8	4.5 ± 0.5	7.7 ± 1.5
ΔPD (30 Cl) (mV)	0.0 ± 0.0	0.1 ± 0.1	8.1 ± 1.3
Ca^{+2}-dep Cl transport	ΣCFTE/con ($n = 6$)	ΣCFTE/T− ($n = 29$)	ΣCFTE/T+ ($n = 3$)
G_{con} (nS)	2.7 ± 0.8	3.7 ± 0.5	2.1 ± 0.3
G_{Ionu} (nS)	5.8 ± 1.7	4.7 ± 0.5	3.4 ± 0.1
Volume dep Cl transport	ΣCFTE/con ($n = 9$)	ΣCFTE/T− ($n = 30$)	ΣCFTE/T+ ($n = 4$)
G_{con} (nS)	2.0 ± 0.4	3.7 ± 0.5	1.8 ± 0.4
G_{Hypo} (nS)	11.3 ± 2.2	19.4 ± 3.4	19.0 ± 1.1

Whole cell conductance (G_{Cl}) was calculated from the measured whole cell current (I) and the applied clamp voltage (V_c). G_{Cl} was determined after stimulation by 10 μM forskolin (G_{For}), by 5×10^{-7} M ionomycin (G_{Ionu}), or by reducing the NaCl concentration in the bath to 72.5 M for hypotonic cell swelling (G_{Hypo}). Conductance of unstimulated control cells (G_{con}) was determined separately for each treatment. Increases in membrane depolarization were determined after reduction of the bath Cl concentration ($\Delta PD_{30\ Cl}$). ΣCFTE/con = forskolin-stimulated nontransfected control cells; ΣCFTE/T− = nonresponding, forskolin-stimulated transfected cells; ΣCFTE/T+ = responding, forskolin stimulated control cells.

replacement and phenotypic correction is close to the range (6–10%) that appears to be sufficient for conversion of a CF epithelial monolayer to one with normal Cl transport properties.[29] These *in vitro* results are encouraging and have been confirmed to some extent in a xenograft model system of an airway epithelium *in vivo*.[19] Furthermore, preliminary analysis in primary airway epithelial cells also indicates that homologous replacement occurs and that *CFTR* mRNA with exogenous sequences is expressed (Z Xu and K Goncz, unpublished data).

From the results of the multiple transfection protocols used, it is clear that each approach for introducing the DNA fragments into the cells is effective in facilitating homologous replacement. There was no obvious effect on the overall ability to detect homologous replacement. Because the CF system does not readily lend itself to quantification at the clonal level, it was difficult to determine whether any particular transfection protocol was more effective than another. It was, however, observed that each transfection protocol (electroporation *versus* gramicidin S–lipid–DNA *versus* dendrimer) showed some variability in the amount of PCR product detected. The cause for this variability is not known and will require further investigation in a system that can be more readily calibrated.

The studies with rec A do not indicate an enhancement of homologous replacement. This may, in part, be due to interference with the encapsulation of the DNA–rec A complex in liposomes or dendrimers. Additional analysis is required to determine whether conditions for rec A-mediated homologous replacement can be optimized or if other recombinases are preferable for enhancement of homologous replacement in human cells.

In summary, SFHR presents an advantage over cDNA gene therapy strategies, because the corrected gene continues to be regulated by endogenous genomic promoters rather than a heterologous enhancer and promoter in the vector. Thus, homologous replacement increases the probability that the corrected gene, whether *CFTR* or another gene, is expressed in the appropriate cells at the appropriate levels. The fact that these studies were carried out in transformed cells could play a role in the overall frequency of homologous replacement detected because of their enhanced proliferative capacity. Although the efficiency of SFHR has not been evaluated in terms of the cell cycle, one could imagine a scenario where replication forks and/or the displacement of Okazaki fragments may facilitate homologous replacement by providing convenient sites of homologous pairing and subsequent gene conversion. Additional studies will need to be carried out in primary cells and at different stages of the cell cycle to test what role cell transformation and proliferation play in homologous replacement.

The finding of functional cAMP-dependent Cl ion transport by patch clamp analysis of gramicidin S–lipid or dendrimer-transfected cells shows promise for application of this technique to the treatment of CF. Whether or not random integration associated with SFHR is limiting in its application to gene therapy also requires further investigation. We are, however, encouraged that these *in vitro* studies may ultimately have important implications for gene therapy in and for development of transgenic animals.

Materials and methods

Cells and culture conditions

Simian virus 40 (SV40)-transformed CF tracheobronchial (ΣCFTE29o−),[29] CF nasal polyp (ΣCFNPE14o−),[31] and transformed CF pancreatic adenocarcinoma (CFPAC-1)[32] and non-CF bronchial (IoHBE14o−)[33] epithelial cells were used. All CF cell lines were homozygous for the *ΔF508* mutation (*ΔF508*/*ΔF508*). The ΣCFTE29o− and

ΣCFNPE14o– cell lines were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics, while the CFPAC-1 cells were grown in Dulbecco's modified Eagle's 'Ham's F12 medium (DME/F12; 1:1). All cell lines were grown under humidified conditions in a 5% CO_2 atmosphere at 37°C.

Fragment preparation

The region of the *CFTR* gene spanning exon 10 (192 bp) and the 3′ (136 bp) and 5′ (165 bp) flanking intron, defined by sequence data published previously,[34] was chosen as the exogenous fragment. A 860-bp fragment including these sequences was generated after cleavage of plasmid T6/20 (ATCC, Rockville, MD, USA)[35] by restriction enzymes *Eco*RI and *Hin*dIII and isolated by electrophoresis in a 0.8% SeaPlaque agarose gel (FMC BioProducts, Rockland, ME, USA).[34,35] A 50-ng aliquot of the 860-bp fragment was used to generate the 491-bp *wtCFTR* fragment by PCR with primers CF1 and CF5 (Figure 1a, Tables 1 and 2). Fragment size was confirmed by electrophoresis on a 1% agarose gel, then amplified in bulk in 20 separate PCR amplifications, each containing 50 ng of template DNA. The 491-bp product was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol. DNA fragments were denatured and 491-nucleotide (nt) ssDNA was used in the targeted replacement studies. The ssDNA fragments were either coated with rec A protein (Pharmacia, Piscataway, NJ, USA)[24,36,37] or left uncoated. Rec A-coated DNA fragments (5 μg in a 4 μl volume) were denatured at 95°C for 10 min, immediately placed in an ice-water bath, then added to 63 μl of reaction buffer containing 200 μg of rec A protein, 4.8 mM ATP-γS, 2 mM Mg-acetate, and 1.7 μl reaction buffer (100 mM Tris-acetate, pH 7.5 at 37°C; 10 mM dithiothreitol; 500 mM Na-acetate) (final volume 67 μl) and incubated for 10 min at 37°C. Next, the Mg-acetate concentration was increased to 20 mM by adding 7 μl of 200 mM Mg-acetate. Under these conditions, the ssDNA fragments were coated with rec A protein at a molar ratio of three bases per one rec A molecule. After coating, the fragments were immediately placed on ice at 4°C until transfection (10 min to 1 h). Fragments left uncoated were taken through the above protocol in the absence of rec A.

Transfection of DNA

In initial studies, cells were transfected with the 491-nt fragments by needle microinjection. Subsequently, fragments were introduced into cells by electroporation, as a gramicidin S-DNA–liposome (GS) complex,[25] or as the polyamidoamine cascade polymer the Starburst dendrimer–DNA (SD) complex.[27]

Electroporation experiments were performed using rec A-coated 491-mer ssDNA as described above. Approximately 10^7 exponentially growing cells were suspended in 400 μl of rec A coating buffer with 5 μg (5 μl) of rec A-coated DNA. The cell suspension was preincubated on ice for 10 min and electroporated at 4°C with 400 V and 400 μF in a BTX 300 electroporator (BTX, San Diego, CA, USA). After electroporation, cells were incubated on ice for an additional 10 min, diluted in Eagle's minimal essential medium (MEM) supplemented as described above, then seeded in a T75 flask. Under these electroporation conditions, approximately 30–50% of the cells survive. Cells were cultured at 37°C in a humidified CO_2 incubator for 5–7 days and then harvested for DNA and RNA.

GS complexes were prepared with dioleoylphosphatidylethanolamine (PtdEtn, DOPE). The lipid was dried under nitrogen at room temperature,[38] rehydrated with 30 mM Tris HCl buffer (pH 9), and then sonicated for 15 min under an argon atmosphere. The complex was prepared in polystyrene tubes by diluting 20 μg of DNA in 30 mM Tris HCl (pH 9) buffer. Gramicidin S was added to the DNA and rapidly mixed. Next, 175 μl of the lipid solution (175 nmol of lipid) was added to the gramicidin S–DNA mixture to create the protein–DNA–lipid complex.

SD complexes were prepared with 4 μg of DNA in rec A coating buffer (45 μl) diluted to a final volume of 330 μl in HEPES buffered saline, 10 mM HEPES, 150 mM NaCl, pH 7.3 (HBS). In a separate polystyrene tube, 25 μg of Starburst dendrimer, sixth generation[27] (in 25 μl, 0.1% w/v) was diluted into a final volume of 170 μl with HBS. The dendrimer solution was then added dropwise to the DNA solution. The transfection cocktail (500 μl per flask) was incubated at room temperature for 5–10 min and then placed into a T25 flask containing cultured cells covered with serum-free medium (1 ml).

Cells were transfected at 70–90% confluence and incubated in serum-free medium with GS or SD complexes (4 μg DNA per dish) for 5 h at 37°C. The medium was then replaced with complete growth medium. Cells were propagated at 37°C after transfection, with daily replacement of medium for 1 week.

DNA and RNA analysis

Genomic DNA and cytoplasmic RNA was isolated and purified from cells as described[30,31,38,39] at different times following transfection. Cellular DNA was initially analyzed for the presence of wt or ΔF sequences following PCR amplification with CF1B/CF6 (Table 1) by allele-specific Southern hybridization. Primers CF1B (sense) and CF6 (antisense) are outside the 5′ and 3′ ends of the homologous region, respectively and give rise to a mixed population wt and *ΔF508* PCR products after amplification of DNA from transfected cells. In addition, CF6 is outside the region defined by the *Eco*RI–*Hin*dIII 860-bp fragment generated from the T6/20 plasmid. This ensures that fragments generated by amplification with CF6 will not detect any residual 860-bp DNA carried through from amplification of the 491-bp fragment. Amplification conditions for CF1B/CF6 were such that the potential for template switching[40,41] should be minimized if not eliminated (Table 2).

Allele-specific Southern hybridization was then used to assess SFHR. The CF1B/CF6 products were hybridized with ^{32}P-labeled oligo N or oligo ΔF probes (Table 1). Hybridization was performed as described previously at 42°C.[30,31,39] After washing, membranes were analyzed autoradiographically by exposure to radiographic film.

Autoradiographs were analyzed with a GS 300 Scanning Densitometer (Hoefer Scientific Instruments, San Francisco, CA, USA) to assess the frequency of homologous replacement. The relative efficiency of hybridization of the oligo N and oligo ΔF probes was determined by comparing the intensity of transmission after hybridization to PCR fragments derived from heterozygote (ΔF/N) lymphocyte controls. For normalization, band